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THE FGF FAMILY OF GROWTH FACTORS AND ONCOGENES

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I. Introduction

During the past few years the family of fibroblast growth factors (FGF) has emerged as perhaps the largest family of growth factors involved in soft-tissue growth and regeneration. It presently includes seven members that share a varying degree of homology at the protein level, and that, with one exception, appear to have a similar broad mitogenic spectrum, i.e., they promote the proliferation of a variety of cells of mesodermal and neuroectodermal origin and are angiogenic. While their genes have similar organization and in some cases map to contiguous regions on human and mouse chromosomes, their pattern of

expression is very different, ranging from extremely restricted expression in some stages of development, to rather ubiquitous expression in a variety of tissues and organs. In addition, some of these factors are efficiently secreted by producer cells, while others are not. They all appear to bind heparin and heparan sulfate proteoglycans and glycosaminoglycans and strongly concentrate in the extracellular matrix. Thus they have also been called heparin-binding growth factors (HBGF) (reviewed in Baird and Bohlen, 1990; Burgess and Maciag, 1989; Rifkin and Moscatelli, 1989).

"Fibroblast growth factor" was originally identified as an activity in pituitary extracts that stimulated the proliferation of mouse 3T3 cells (Armelin, 1973; Gospodarowicz, 1974). The mitogenic activity was partially purified from bovine pituitary and brain and was found to be due to a molecule with a molecular weight of 14K–16K with a basic isoelectric point (Gospodarowicz, 1975; Gospodarowicz *et al.*, 1978a). Shortly afterward evidence accumulated that a second 3T3 cell mitogen with an acidic isoelectric point occurred in brain (Maciag *et al.*, 1979; Thomas *et al.*, 1980). The mitogenic activity of these FGFs was not restricted to fibroblasts, and stimulated many cell types, including endothelial cells and chondrocytes (Gospodarowicz *et al.*, 1978b). Although small amounts of both the basic and the acidic mitogen were purified by conventional chromatography methods (Bohlen *et al.*, 1984; Thomas *et al.*, 1984), further characterization was aided by observations made by investigators purifying angiogenesis factors, agents that stimulate new blood vessel growth. Since both angiogenic factors and the FGFs were mitogenic for endothelial cells, they were suspected to be closely related. The discovery that an endothelial cell mitogen from a tumor extract bound strongly to heparin (Shing *et al.*, 1984) suggested that heparin affinity columns might be used for the purification of the FGFs. Indeed, both basic and acidic FGFs bound strongly to heparin affinity columns, with acidic FGF eluting at 1 *M* NaCl and basic FGF eluting at 1.5 *M* NaCl (Gospodarowicz *et al.*, 1984; Klagsbrun and Shing, 1985; Maciag *et al.*, 1984). Complete characterization of the FGFs was greatly facilitated by the use of heparin affinity columns, which, by permitting a simple and rapid purification of FGFs, also helped demonstrate that the mitogenic and angiogenic activities identified in a variety of tissues were mostly due to either basic or acidic FGF. This reduced a large list of poorly characterized mitogens to synonyms for basic and acidic FGF.

However, the apparent simplicity of the FGF family did not last, and the family has grown to seven members. Three other members of the family (K-FGF/HST, FGF-5, INT-2) were identified originally as oncogenes, while the two latest additions, FGF-6 and keratinocyte growth

factor (KGF), were isolated by sequence homology or factor purification and cloning. The related molecules that have been characterized have mitogenic activity and also bind tightly to heparin affinity columns. Further complexity arises from the fact that some members of the family also exist in multiple forms arising from initiation of translation at alternative codons (Table I).

Genes or proteins corresponding to the same FGF often appear in the literature under different names, reflecting their origin, isolation, etc. To alleviate this cause of confusion, a new nomenclature has been proposed (Aaronson *et al.*, 1991). In this proposal FGFs are numbered consecutively (i.e., acidic FGF = FGF-1, basic FGF = FGF-2, etc.). Also, this proposal recommends that the name fibroblast growth factor, which implies a spectrum of action specific for fibroblasts, be discontinued and replaced simply by FGF. We have continued in this article to use the old nomenclature, but we have indicated also the proposed new names. The proposal for a new nomenclature also suggests a numbering system for the amino acids of the FGFs. This numbering system designates the amino acid immediately following the initiation methionine as amino acid 1. We have adopted the new numbering system in the discussion of FGF structure.

The genes and cDNAs for most FGFs have been cloned, often in difference species, and some knowledge about their receptors is starting to emerge. In spite of the large body of information that is accumulating about these growth factors, little is known about their physiological and pathological role. While in tissue culture they stimulate the growth and proliferation of a wide variety of cell types and often seem to exhibit an identical spectrum of action, it is not clear whether this apparent lack of specificity is also reflected *in vivo*.

In a similar vein, although it is clear that some of the FGFs can be potently oncogenic in model systems, their involvement in human tumors remains to be clarified. We will review in this article the protein structure, localization, gene organization, and regulation of expression of these growth factors in an attempt to understand their mechanism of action, interaction with their receptors, and ultimately their function and role in physiology and development, as well as in pathological conditions.

II. Protein Structure

A. Basic FGF (FGF-2)

Basic FGF (bFGF) was originally purified from bovine pituitary as a 146-amino acid protein with a molecular weight of 16.5K and an iso-

TABLE I

PROPERTIES OF HUMAN FGFs[a]

Growth factor	Primary translation product[b]	Size of secreted form[b]	Heparin affinity[c]	Known high-affinity receptors	Gene mapping (human)
aFGF (FGF-1)	155	Not secreted	1.0 *M*	FGFR-1 R-2, R-3, R-4	5q31–33
bFGF (FGF-2)	155	Not secreted	1.6 *M*	FGFR-1, FGFR-2	4q25
	196[d]				
	201[d]				
	210[d]				
INT-2 (FGF-3)	239	ND[e]	ND[e]		11q13
	271[d]				
K-FGF/HST (FGF-4)	206	176	1.2–1.3 *M*	FGFR-1, FGFR-2	11q13
FGF-5	267	ND[e]	1.0–1.5 *M*		4q21
FGF-6	198 (murine)	ND[e]	ND[e]		12p13
KGF (FGF-7)	194	~163	0.6 *M*	FGFR-2 (variant)	

[a] Unless otherwise noted, data are for human FGFs. Pertinent references are in the text.
[b] Number of amino acids.
[c] NaCl concentration required for elution from heparin affinity columns.
[d] Products of translation initiation at upstream CUG.
[e] ND, Not determined.

electric point of 9.6 (Esch *et al.*, 1985a). Molecules with identical properties were purified from bovine brain, retina, and adrenal, and from human brain (Baird *et al.*, 1985; Bohlen *et al.*, 1985; Gimenez-Gallego *et al.*, 1986; Gospodarowicz *et al.*, 1984, 1986). Smaller forms containing truncations at the amino-terminal end, but which still retained biological activity, were isolated from corpus luteum, adrenal, and testes (Gospodarowicz *et al.*, 1985; Ueno *et al.*, 1987). It is not clear whether all of these forms occur *in vivo*. At least some of the truncated forms seem to arise from proteolysis that occurs during the extraction procedure (Klagsbrun *et al.*, 1987), and, with careful isolation in the presence of protease inhibitors, bFGF molecules even larger than 146 amino acids have been obtained (Story *et al.*, 1987; Ueno *et al.*, 1986). From these results, it is unclear how much proteolytic processing of bFGF occurs *in vivo*.

When both bovine and human bFGF cDNAs were cloned, an AUG codon was found in the proper context to initiate translation of a protein of 155 amino acids, and no in-frame AUG codons were found upstream (Abraham *et al.*, 1986a,b). Therefore, translation was predicted to initiate at this AUG codon and to result in an 18-kDa protein. However, larger forms of bFGF were purified from human placenta and from guinea pig brain (Moscatelli *et al.*,1987; Sommer *et al.*, 1987). These higher molecular weight forms of bFGF have been shown to arise from the use of three upstream CUG codons as alternate initiation codons for translation. Thus, human bFGF is expressed in four forms, an 18-kDa form (155 amino acids) generated by initiation at the AUG codon and 22-, 22.5-, and 24-kDa forms (196, 201, and 210 amino acids) arising from the CUG codons (Florkiewicz and Sommer, 1989; Prats *et al.*, 1989). The high molecular weight forms of bFGF contain the same amino acid sequence as the 18K form but have additional N-terminal extensions of varying lengths. Both 18K and higher molecular weight forms are expressed in brain (Moscatelli *et al.*, 1987; Presta *et al.*, 1988) and a number of different cell lines (Ensoli *et al.*, 1989a; Iberg *et al.*, 1989; Renko *et al.*, 1990; Tsuboi *et al.*, 1990).

The different forms of bFGF seem to be correlated with differences in subcellular distribution. Both the 155-amino acid bFGF and the higher molecular weight forms lack a typical signal sequence for secretion and seem to be retained within the cell. The 155-amino acid form is primarily located in the cytosol while the higher molecular weight forms are present in the nuclear and ribosomal fractions (Renko *et al.*, 1990; Florkiewicz *et al.*, 1991). These results suggest that the higher molecular weight forms of bFGF contain a nuclear translocation sequence. Indeed, when the N-terminal extension of the higher molecular weight forms is

grafted to normally cytoplasmic proteins, it is able to drive these recombinant proteins to the nucleus (Bugler *et al.*, 1991; Quarto *et al.*, 1991a). The N-terminal extensions in the higher molecular weight forms of bFGF contain several stretches of alternating glycine and arginine residues. Some of the arginine residues in these sequences are methylated (Burgess *et al.*, 1991; Sommer *et al.*, 1989), as has been described for other nuclear proteins. The significance of nuclear forms of bFGF is presently not clear.

In addition to endogenous nuclear forms of bFGF, translocation of exogenous 18-kDa bFGF to the nucleus has been described (Bouche *et al.*, 1987). This translocated bFGF associates primarily with the nucleolus. A putative nuclear translocation sequence has also been identified in amino acids 26 to 31. Mutant 18-kDa bFGF lacking this sequence retains full mitogenic activity but has a reduced ability to induce plasminogen activator in endothelial cells (Isacchi *et al.*, 1991). While all of these findings are very provocative, a conclusive interpretation of these data will require additional work.

The amino acid sequence of 18-kDa bFGF is highly conserved among species with 89–95% identity among human, bovine, ovine, and rat bFGFs (Abraham *et al.*, 1986a,b; Kurokawa *et al.*, 1988; Shimasaki *et al.*, 1988; Simpson *et al.*, 1987). *Xenopus* bFGF is more divergent, but still shares ~80% homology with human bFGF (Kimelman *et al.*, 1988). This low level of divergence suggests that there may be functional importance for all regions of bFGF. There seems to be more variation in the N-terminal extensions of the high molecular weight forms of bFGF (Brigstock *et al.*, 1990; Sommer *et al.*, 1989), suggesting that these regions are less functionally restricted. Additional information about biologically active sequences in bFGF can be obtained by comparing it to other members of the FGF family. However, the homology to other members of the family extends over almost the entire sequence of bFGF, from amino acids 27 to 149. bFGF contains four cysteine residues, and two of these are conserved among all members of the FGF family, suggesting that they may have important functions in the biology of the FGFs. However, none of the cysteines are strictly necessary for biological activity, since *in vitro* mutagenesis of the cysteine residues to serine does not alter the mitogenic activity of bFGF (Fox *et al.*, 1988; Seno *et al.*, 1989; Arakawa *et al.*, 1989).

The heparin-binding and receptor-binding regions of bFGF have been mapped based on the ability of synthetic peptides representing different amino acid sequences in bFGF to bind radiolabeled heparin, to block binding of radiolabeled bFGF to its receptor, and to act as agonists or antagonists of bFGF biological activity (Baird *et al.*, 1987, 1988). The

receptor-binding activity was found in two regions, amino acids 32–76 and 114–123. The inclusion of C-terminal sequences in the peptide containing amino acids 114–123 increased its potency. In these studies, heparin binding was strongest in these same regions, but lower heparin-binding capacity was found in other sequences, suggesting that heparin-binding sites are distributed throughout the molecule. Other experiments suggest that the receptor-binding and heparin-binding regions of bFGF are distinct. Antibodies that bind to bFGF and inhibit its interaction with its receptor have no affect on the heparin affinity of the molecule (Kurokawa *et al.*, 1989). Furthermore, Seno *et al.* (1990) found that deleting 42 amino acids from the C terminus of bFGF abolished the heparin affinity of bFGF, but the molecule still retained some biological activity, although it was about 10^4 times less potent than the intact molecule. These studies also showed that mutant forms of bFGF lacking amino acids 1–48 had normal affinity for heparin, while forms lacking more than 6 amino acids from the C terminus had reduced affinity for heparin, suggesting that the carboxy-terminal structure was important for heparin binding (Seno *et al.*, 1990). X-Ray crystalographic studies of the three-dimensional structure of bFGF show a cluster of basic residues in this region. In addition, the crystals contain ordered sulfate ions forming ionic contacts with Lys-127, Lys-137, Lys-133, and Arg-128, which may mimic the contacts made by sulfate moieties in heparin (Eriksson *et al.*, 1991; Zhang *et al.*, 1991). The major structural characteristics of bFGF are shown in Fig. 1.

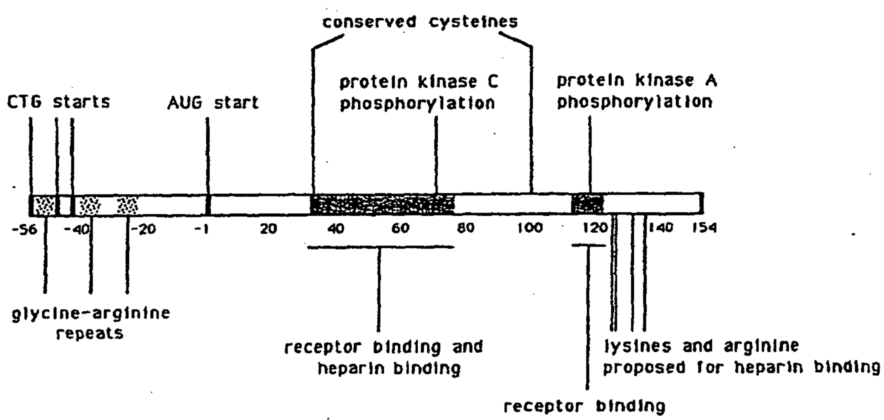

FIG. 1. Schematic representation of the bFGF molecule. The schematic diagram represents the amino acid sequence of both 18K and higher molecular weight bFGFs. The scale on the bottom marks the location of the amino acid residues with the methionine that initiates 18K bFGF designated amino acid −1. The relative locations of structural features described in the text are indicated.

bFGF has also been reported to be a substrate for phosphorylation by protein kinase C and protein kinase A (Feige and Baird, 1989). Phosphorylation by protein kinase C occurs on Ser-72 while phosphorylation by protein kinase A occurs at Thr-120. Phosphorylation by protein kinase A occurs in one of the putative receptor-binding regions and seems to slightly increase the affinity of bFGF for its receptor, while phosphorylation by protein kinase C has no effect on receptor affinity. Although the biological significance of phosphorylation of bFGF is not clear, phosphorylated bFGF has been identified in extracts of cultured endothelial cells, and, thus, appears to occur naturally in these cells.

The cellular source of bFGF is uncertain. It has been found in all organs and tissues examined except serum (Baird *et al.*, 1986). It is synthesized by cultured fibroblasts, endothelial cells, glial cells, and smooth muscle cells (Connolly *et al.*, 1987; Gospodarowicz *et al.*, 1988; Hatten *et al.*, 1988; Moscatelli *et al.*, 1986a; Schweigerer *et al.*, 1987a; Vlodavsky *et al.*, 1987; Weich *et al.*, 1990), and, since these cell types are ubiquitous, they may be the source of bFGF in the organs.

B. Acidic FGF (FGF-1)

Acidic FGF (aFGF) was isolated originally as a 154-amino acid protein in addition to truncated forms of 140 and 134 amino acids (Burgess *et al.*, 1986; Esch *et al.*, 1985b; Gimenez-Gallego *et al.*, 1985; Harper *et al.*, 1986). The N terminus of the 154-amino acid form is acetylated (Crabb *et al.*, 1986), but acetylation seems to have no effect on the biological activity of the molecule. Several groups have shown that recombinant nonacetylated aFGF made in bacteria, recombinant acetylated aFGF made in yeast, and natural aFGF all have equivalent mitogenic and angiogenic potencies (Barr *et al.*, 1988; Jaye *et al.*, 1987; Linemeyer *et al.*, 1987).

The primary translation product for aFGF is, however, a 155-amino acid protein (Jaye *et al.*, 1986). There appear to be no N terminal-extended forms of aFGF, since a termination codon is found at position −1 to the AUG initiation codon. However, the existence of alternate 5′-untranslated exons in aFGF messengers has been described (Chiu *et al.*, 1990; Crumley *et al.*, 1990). The role of these untranslated sequences is unknown but might be involved in the differential regulation of translation of the molecule. The aFGF protein, like bFGF, has no signal sequence for secretion and is inefficiently released by cells that product it.

aFGF has 55% amino acid sequence identity to 18-kDa bFGF. Homology extends over the entire sequence of the molecule except for the 18 N-terminal amino acids and a 2-amino acid insert at positions 116 and

117. aFGF contains three cysteine residues that, like the cysteine residues in bFGF, do not seem to be necessary for biological activity. Crabb *et al.* (1986) reported that aFGF in which these residues have been reduced and carboxymethylated still retains biological activity. In contrast, Jaye *et al.* (1987) have reported that quantitative alkylation of cysteine residues abolishes the receptor-binding activity. Finally, the studies of Linemeyer *et al.* (1990) have shown that disulfide bond formation between the cysteines drastically reduces the biological activity of aFGF. These studies showed that cysteine residues in brain-derived and recombinant aFGF are normally reduced, that formation of intramolecular disulfide bonds results in an inactive molecule, and that reduction of the disulfide bonds restores activity. In addition, site-directed mutation of any of the cysteine residues to serine results in an aFGF with high biological activity (Linemeyer *et al.*, 1990). Indeed, substitution of any two of the three cysteines with serine residues produces aFGF that is less heparin dependent and more stable in the absence of heparin (Ortega *et al.*, 1991). Together these investigations suggest that the cysteine residues are not necessary for biological activity, but modification of the cysteines may hinder the formation of biologically active conformations of aFGF.

Immunolocalization studies have also identified nuclear forms of aFGF (Sano *et al.*, 1990; Speir *et al.*, 1991). A putative nuclear localization sequence has been identified in residues 21–27 of the protein. Deletion of this sequence results in a molecule of reduced potency, while substitution of the nuclear translocation sequence from yeast histone 2B restores biological activity (Imamura *et al.*, 1990). Although this basic sequence seems to be necessary for full biological activity of aFGF, its role as a nuclear targeting sequence has not been directly demonstrated. Thus, it is not clear whether the biological effects of deletion of this sequence are mediated through effects on the ability of aFGF to be translocated to the nucleus.

Attempts to map binding regions of aFGF have identified two sites. A synthetic peptide corresponding to residues 49–72 of the primary translation product competed with aFGF for heparin binding (Mehlman and Burgess, 1990). This region is homologous to one of the regions identified for heparin binding in bFGF. A second binding region was indicated by chemical modification experiments. Methylation of lysine residues in aFGF caused a reduction in its affinity for heparin, receptor affinity, and biological potency (Harper and Lobb, 1988). The alteration in activity was correlated with the modification of Lys-132. Site-directed mutation of this lysine to glutamic acid resulted in an aFGF with a lower affinity for heparin and reduced mitogenicity but with no alteration in its receptor affinity or ability to stimulate early responses in cells (Burgess *et al.*,

1990a). Thus, this residue seems to be important in heparin interactions but not in receptor binding.

C. INT-2 (FGF-3)

The INT-2 gene was originally identified as a site of frequent insertion of the mouse mammary tumor virus (Dickson *et al.*, 1984). Viral insertion activated the transcription of the cellular gene, leading to tumor formation. The predicted product of this gene, the INT-2 protein, is 245 amino acids in the mouse or 239 amino acids in humans (Brookes *et al.*, 1989a). The core of the protein has 44% amino acid sequence homology to bFGF, while the N-terminal and C-terminal sequences are unique (Dickson and Peters, 1987). The protein contains a short stretch of hydrophobic amino acids at the N terminus that may serve as an atypical secretory signal sequence. Indeed, INT-2 protein has been detected in the endoplasmic reticulum and Golgi compartments (Acland *et al.*, 1990), but release into the medium seems to be inefficient. The primary translation product has a molecular weight of 28.5K, and posttranslational modifications, including glycosylation and a presumed cleavage of the signal peptide, give rise to 30.5K and 31.5K forms (Dixon *et al.*, 1989). INT-2 protein can also initiate from a CUG codon upstream from the normal AUG codon, giving rise to a 271-amino acid human protein or a 274-amino acid (31.5 kDa) mouse protein (Acland *et al.*, 1990). The N-terminal extended form of INT-2, like the N-terminal extended forms of bFGF, is localized in the nucleus.

INT-2 seems to be expressed primarily during development, and has not been detected in any normal adult tissue.

D. K-FGF/HST (FGF-4) AND FGF-6

The HST/K-FGF gene was discovered by screening for genes present in human stomach tumors or Kaposi's sarcoma that are able to transform NIH 3T3 cells (Delli Bovi and Basilico, 1987; Sakamoto *et al.*, 1986). The human HST/K-FGF gene encodes a 206-amino acid protein (K-FGF) with a classical signal sequence for secretion (Delli Bovi *et al.*, 1987; Taira *et al.*, 1987). The murine K-FGF protein is only 202 amino acids long, but otherwise has about 85% identity to the human protein (Brooks *et al.*, 1989b). While the N-terminal 80 amino acids of the protein are unique, the remaining 126 amino acids of the human protein have about 40% homology to human bFGF. Thirty or 31 N-terminal amino acids containing the signal sequence are cleaved during posttranslational processing, giving rise to a final product of 175 or 176 amino acids (Delli Bovi *et al.*,

1988). The protein contains one site for N-linked glycosylation, and glycosylation increases the molecular weight to about 23K (Delli Bovi *et al.*, 1988). The protein is efficiently secreted by cells that express it. K-FGF contains the two conserved cysteines of all FGFs and also in this case they do not appear essential for function (D. Talarico and C. Basilico, unpublished).

K-FGF seems to be expressed only at limited times during development. In animal models, inappropriate expression of the growth factor in adult tissues leads to tumor formation through autocrine activation of FGF receptors on the cell surface (Talarico *et al.*, 1990; Talarico and Basilico, 1991).

The FGF-6 gene was discovered after screening a library with probes to the HST/K-FGF gene (Marics *et al.*, 1989). The FGF-6 protein is closely related to K-FGF and seems to be structurally very similar. Little is known about the biology of FGF-6, but it has also been shown to be a secreted protein (deLapeyriere *et al.*, 1990).

E. FGF-5

The FGF-5 gene also was identified by screening for genes present in tumors that are able to transform NIH 3T3 cells (Zhan *et al.*, 1988). The gene encodes a 267-amino acid protein. Like INT-2, the central core of the protein has about 50% homology to bFGF, but the N-terminal and C-terminal sequences are unique. The molecule contains a classical signal sequence and is efficiently secreted. The molecular weight of the primary translation product is 29.5K. Posttranslational processing, including cleavage of the signal sequence, N-linked glycosylation at one site, and possible O-linked glycosylation, yields molecules of 32.5–38.5 kDa (Bates *et al.*, 1991).

FGF-5 is expressed in a site-specific manner at limited times during development. No FGF-5 mRNA could be detected in extracts of a number of adult tissues, but low levels of FGF-5 mRNA have been detected in localized areas of adult brain by *in situ* hybridization (Haub *et al.*, 1990).

F. Keratinocyte Growth Factor (FGF-7)

Keratinocyte growth factor (KGF) was isolated as a mitogen for a cultured murine keratinocyte line (Rubin *et al.*, 1989). Unlike the other members of the FGF family, it has little activity on mesenchyme-derived cells but stimulates the growth of epithelial cells. The keratinocyte growth factor gene encodes a 194-amino acid polypeptide (Finch *et al.*, 1989). The N-terminal 64 amino acids are unique, but the remainder of

the protein has about 30% homology to bFGF. All in all, KGF is the most divergent member of the FGF family. The molecule has a hydrophobic signal sequence and is efficiently secreted. Posttranslational modifications include cleavage of the signal sequence and N-linked glycosylation at one site, resulting in a protein of 28 kDa.

Keratinocyte growth factor is produced by fibroblasts derived from skin and fetal lung (Rubin *et al.*, 1989). The keratinocyte growth factor mRNA was found to be expressed in adult kidney, colon, and ilium, but not in brain or lung (Finch *et al.*, 1989). The conserved regions within the FGF protein family are shown in Fig. 2.

III. The FGF Genes and Their Expression

Consistent with their origin from a common ancestral gene, the FGF genes studied so far appear to have a similar organization. They consist of three exons, separated by two introns of variable length. Typically the second exon is very short and in many cases the third exon includes a very long (2–3 kb) 3′-untranslated sequence. Although we did not conduct a detailed analysis, there seems to be very little homology at the DNA level among FGF genes outside of the coding regions.

The FGF genes map on several chromosomes. The two prototypes of the family, basic and acidic FGF, map on human chromosomes 4q25 and 5q31–33, respectively (Fukushima *et al.*, 1990; Jaye *et al.*, 1986). INT-2 and K-FGF are very closely linked on 11q13 (Huebner *et al.*, 1988; Nguyen *et al.*, 1988; M. C. Yoshida *et al.*, 1988). In the mouse, these genes are arranged head to tail and separated only by about 20 kb of DNA (Brookes *et al.*, 1989b). FGF-5 maps on human chromosome 4q21 (Nguyen *et al.*, 1988) and FGF-6 on 12p13 (Marics *et al.*, 1989). FGF genes have been found in all mammals, birds, and amphibians. We are not aware of any report of FGF-like genes in *Drosophila* or yeast. It is likely that FGF-like molecules exist in *Drosophila* and may have a role in development, because a putative *Drosphila* FGF receptor has just been reported (Glazer and Shilo, 1991).

The pattern of expression of each FGF family member is quite distinct. Basic and acidic FGF are quite ubiquitous and are present in most tissues at relatively high concentration, generally bound to the extracellular matrix (ECM). At the RNA level, their expression is particularly high in the brain. Presumably these factors are produced at low levels by a variety of tissues and cells, and accumulate in the ECM. As already mentioned, the situation is further complicated by the fact that bFGF and aFGF do not possess a signal peptide and are very inefficiently secreted by producer cells.

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K-FGF   MS.GPGTAAV ALLPAVLLAL LA........ .PWAGRGGAA APTAPNGTLE   40
FGF-6   MSRGAGRVQG TLQALVFLGV LV........ .GMVVPSPAG AR..ANGTLL   39
FGF-5   .......MSL SFLLLLFFSH LILSAWAHGE KRLAPKGQPG PAATDRNPIG   43
aFGF    .......... .......... .......... .......... ..........
bFGF    .......... .......... .......... .......... ..........
KGF     .......... .......... .......MHK WILTWILPTL LYRSCFHIIC   23
INT-2   .......... .......... .......... .......... .........M    1

K-FGF   AELERRWESL VALSLARLPV AAQPKEAAVQ SGAGDYLLGI .KRLRRLYCN   89
FGF-6   D..SRGWGTL ..LSRSRAGL AGEISGVNWE SG...YLVGI .KRQRRLYCN   81
FGF-5   SSSRQSSSSA MSSSSASSSP AASLGSQGSG LEQSSFQWSL GARTGSLYCR   93
aFGF    .......... .....MAEGE ITTFTALTEK FN...LPPGN YKKPKLLYCS   32
bFGF    .......... .....MAAGS ITTLPALPED GGSGAFPPGH FKDPKRLYCK   35
KGF     LVGTISLACN DMTPEQMATN VNCSSPERHT RSYDYMEGGD IRV.RRLFCR   72
INT-2   GLIWLLLLSL LEPGWPAAGP GARLRRDAGG RGGVYEHLGG APRRRKLYCA   51

K-FGF   VGIGFHLQAL PDGRIGGAHA .DTRDSLLEL SPVERGVVSI FGVASRFFVA  138
FGF-6   VGIGFHLQVP PDGRISGTHE .ENPYSLLEI STVERGVVSL FGVKSALFIA  130
FGF-5   VGIGFHLQIY PDGKVNGSHE .ANMLSVLEI FAVSQGIVGI RGVFSNKFLA  142
aFGF    NG.GHFLRIL PDGTVDGTRD RSDQHIQLQL SAESVGEVYI KSTETGQYLA   81
bFGF    NG.GFFLRIH PDGRGDGVRE KSDPHIKLQL QAEERGVVSI KGVCANRYLA   84
KGF     T..QWYLRID KRGKVKGTQE MKNNYNIMEI RTVAVGIVAI KGVESEFYLA  120
INT-2   T..KYHLQLH PSGRVNGSLE NS.AYSILEI TAVEVGIVAI RGLFSGRYLA   98

K-FGF   MSSKGKLYGS PFFTDECTFK EILLPNNYNA YESYKYPGM. ..........  177
FGF-6   MNSKGRLYTT PSFHDECKFR ETLLPNNYNA YESDLYRGT. ..........  169
FGF-5   MSKKGKLHAS AKFTDECKFR ERFQENSYNT YASAIHRTEK TG........  184
aFGF    MDTDGLLYGS QTPNEECLFL ERLEENHYNT YISKKHAEKN ..........  121
bFGF    MKEDGRLLAS KCVTDECFFF ERLESNNYNT YRSRKYT..S ..........  122
KGF     MNKEGKLYAK KECNEDCNFK ELILENHYNT YASAKWTHNG GE........  162
INT-2   MNKRGRLYAS EHYSAECEFV ERIHELGYNT YASRLYRTVS STPGARRQPS  148

K-FGF   .....FIALS KNGKTKKG.. NRVSPTMKVT HFLPRL.... ..........  206
FGF-6   .....YIALS KYGRVKRG.. SKVSPIMTVT HFLPRI.... ..........  198
FGF-5   ..REWYVALN KRGKAKRGCS PRVKPQHIST HFLPRFKQSE QPELSFTVTV  232
aFGF    ....WFVGLK KNGSCKRG.. PRTHYGQKAI LFLPLPVSSD ..........  155
bFGF    ....WYVALK RTGQYKLG.. SKTGPGQKAI LFLPMSAKS. ..........  155
KGF     ....MFVALN QKGIPVRG.. KKTKKEQKTA HFLPMAIT.. ..........  194
INT-2   AERLWYVSVN GKGRPRRG.. FKTRRTQKSS LFLPRVLDHR DHEMVRQLQS  196

K-FGF   .......... .......... .......... .......... ...
FGF-6   .......... .......... .......... .......... ...
FGF-5   PEKKNPPSPI KSKIPLSAPR KNTNSVKYRL KFRFG..... ...  267
aFGF    .......... .......... .......... .......... ...
bFGF    .......... .......... .......... .......... ...
KGF     .......... .......... .......... .......... ...
INT-2   GLPRPPGKGV QPRRRRQKQS PDNLEPSHVQ ASRLGSQLEA SAH  239
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FIG. 2. Protein sequence homology within the FGF family. The deduced amino acid sequences of the seven FGFs are aligned with some conserved motifs highlighted. The arrows bracket the conserved "core" region common to all FGFs. Sequences are for human FGFs, with the exception of FGF-6, which is murine. The N-terminal extensions produced by CUG initiation of bFGF and INT-2 mRNAs are not shown.

bFGF has been found in most tissues and organs examined. A variety of cultured cells, including a number of tumor cell lines (Klagsbrun *et al.*, 1986; Moscatelli *et al.*, 1986a; Presta *et al.*, 1986; Schweigerer *et al.*, 1987b), synthesize bFGF, suggesting that expression of bFGF is widespread *in vivo*. There is some evidence that expression of bFGF increases as an adaptation to culture (Speir *et al.*, 1991), raising the possibility that conclusions about the source of bFGF based on cultured cells may be misleading. However, immunolocalization studies of normal adult tissue have shown that bFGF is indeed produced *in vivo* by a variety of cell types, including skeletal, cardiac, and smooth muscle, and epithelial cells of sweat glands, trachea, bronchi, colon, and endometrial glands (Cordon-Cardo *et al.*, 1990). bFGF is also found in the endothelial cells of some blood vessels, but not in all (Cordon-Cardo *et al.*, 1990; Hanneken *et al.*, 1989). In the adult, the mRNA is expressed in much higher levels in brain than in other tissues (Shimasaki *et al.*, 1988). In normal brain, high-level expression is restricted to discrete areas, where it is present mainly in neuronal cell bodies, but lower level expression is found throughout the tissue, where it seems to be associated with glial cells (Emoto *et al.*, 1989; Finklestein *et al.*, 1988; Pettmann *et al.*, 1986). In injured brain the expression of bFGF increases in the area of injury and seems to be associated with reactive astrocytes (Finklestein *et al.*, 1988).

aFGF appears to have a more limited distribution than bFGF. It has been found in neural tissue, kidney, prostate, and cardiac muscle (Casscells *et al.*, 1990; Crabb *et al.*, 1986; D'Amore and Klagsbrun, 1984; Gautschi-Sova *et al.*, 1987; Quinkler *et al.*, 1989; Sasaki *et al.*, 1989; Thomas *et al.*, 1984). Immunolocalization studies have detected aFGF in neurons in discrete regions of the cerebrum and cerebellum (Wilcox and Unnerstall, 1991). It has also been identified in cultured vascular smooth muscle cells (Weich *et al.*, 1990; Winkles *et al.*, 1987).

The physiological distribution of INT-2 is much more restricted. INT-2 is generally not expressed in adult tissues, including the mammary gland, although it appears to be produced in precise steps of embryo development (Jakobovits *et al.*, 1986; Wilkinson *et al.*, 1988). INT-2 transcripts were detected by *in situ* hybridization in extraembryonic endoderm, localized to the parietal endoderm. INT-2 RNA is also present in cells of the primitive streak and later detected in the hind brain and pharingeal pouches. Sometime beyond day 10 of gestation INT-2 expression appears to be switched off. Similarly, K-FGF is also undetectable in adult tissues and in "normal" cell lines (Hebert *et al.*, 1990; Velcich *et al.*, 1989), but is present in the mouse blastocyst (inner cell mass), primitive streak, and myotomes (Niswander and Martin, 1992). Interestingly, INT-2 and K-FGF have a mirror-like pattern of expression in embryonal

carcinoma (EC) cells. K-FGF is expressed in undifferentiated EC cells, and induction of differentiation shuts off its expression (Curatola and Basilico, 1990; Velcich *et al.*, 1989). INT-2 is transiently turned on after induction of differentiation (Jakobovits *et al.*, 1986). Thus the pattern of expression of these two genes suggest that their physiological role may be related to development.

In *Xenopus* embryonic development, both bFGF and K-FGF appear to be expressed (Kimelman *et al.*, 1988; Slack and Isaacs, 1989; J. C. Slack, personal communication). Low levels of maternal messages are present, and they increase in abundance at the blastula level. Both these factors can induce mesoderm formation in *Xenopus* animal pole explants, strongly suggesting a role for FGFs in mesodermal induction (Kimelman *et al.*, 1988; Paterno *et al.*, 1989; Slack *et al.*, 1987).

FGF-5 is also expressed at specific development stages in the mouse embryo (Haub and Goldfarb, 1991), but is also present in adult brain (Haub *et al.*, 1990) and many "normal" and tumor cell lines (Zhan *et al.*, 1988). During development, FGF-5 RNA is not detected in the blastocyst, but it later appears in the postimplantation epiblast, splanchnic mesoderm, somatic mesoderm, myotomes, limb mesenchymes, and acoustic ganglia (days 12–14). Interestingly, at several of these sites, expression is spatially restricted within the tissue (Haub and Goldfarb, 1991). Thus, although it appears that FGF-5 may be in some cases expressed at similar times and sites as INT-2 and K-FGF, it is quite possible that different cells in the developing organs express different types of FGFs.

Thus, these observations strongly suggest a role for INT-2, K-FGF, and FGF-5 in development, but the precise meaning of these findings remains to be elucidated. Not much is known yet about FGF-6 and KGF, except that the latter is produced by a variety of epithelial tissues and stromal cells.

Molecular Regulation of FGF Expression

The cis- and trans-acting elements involved in regulating FGF transcription are just beginning to be elucidated.

Perhaps the FGF gene whose control of transcription is best known is K-FGF. The K-FGF gene has a canonical TATA box located about 30 nucleotides upstream of the transcription initiation site. However, the K-FGF promoter and upstream DNA sequences can promote only very low levels of transcription when placed upstream of a reporter gene [such as chloramphenicol acetyltransferase (CAT)] on transfection in a variety of cell types, including undifferentiated EC cells, which express high levels

of endogenous K-FGF transcripts (Curatola and Basilico, 1990). Juxtaposition of generalized enhancers [such as that of simian virus 40 (SV40)] to the K-FGF promoter results in ubiquitous, nonspecific expression, and a similar mechanism (juxtaposition of general enhancer elements) is likely to be responsible for activation of the K-FGF oncogene. Physiological K-FGF transcription depends instead on the presence of an enhancer element that is located in the 3'-untranslated region of the third exon and is present in a comparable location in the murine and human gene (Curatola and Basilico, 1990). This enhancer promotes transcription only in undifferentiated EC cells, but not in their differentiated counterpart or in HeLa or 3T3 cells, thus mimicking the pattern of expression of the endogenous gene. This indicates that the K-FGF enhancer interacts with specific trans-acting factors whose expression is also developmentally regulated.

The mouse K-FGF enhancer has been narrowed down to a minimum 270-nucleotide fragment with full activity that contains a series of consensus binding sites for several known transcription factors, including Spl and AP4. Site-directed mutagenesis of these binding sites decreases somewhat enhancer strength, but mutagenesis of an octamer-binding site completely abolishes enhancer activity (A. M. Curatola, Daaka, Dailey, and C. Basilico, unpublished). Thus the critical factor(s) for K-FGF transcription are likely to belong to the family of octamer-binding proteins, some of which have been recently known to be developmentally regulated (Scholer *et al.*, 1989).

The K-FGF transcript is a single RNA species of 3.4 Kb (Velcich *et al.*, 1989). There is no evidence of posttranscriptional or translational control. An mRNA of about 1.1 kb is, however, strongly expressed in 3T3 cells transfected with the K-FGF oncogene as originally isolated (Delli Bovi *et al.*, 1987). This RNA is identical to the physiological RNA species in the coding region, but is prematurely terminated approximately 250 nucleotides downstream of the translation stop codon. This is likely to result from the 3' rearrangement found in the K-FGF oncogene, which occurs upstream of and eliminates the normal termination site (Delli Bovi and Basilico, 1987). The 1.1-kb RNA is somewhat more stable than the normal transcript, and this could contribute to the activation of the K-FGF oncogene, which, however, appears to be mainly transcriptional. The protein is efficiently secreted. Thus the main regulation of this gene appears to be transcriptional.

Not much is known about transcriptional regulation of the bFGF and aFGF genes. Both genes produce multiple transcripts, likely to result from alternative splicing and polyadenylation (Abraham *et al.*, 1986b; Crumley *et al.*, 1990). The human bFGF promoter does not appear to

have a TATA box, but contains several potential SP1 and one AP1 binding site. Negative regulatory elements, whose deletion increases gene expression, appear to be present 5′ of the promoter region (Shibata *et al.*, 1991). As mentioned above, the bFGF mRNA can be translated from four initiation sites: a canonical AUG, which results in the synthesis of the 155-amino acid protein, and three upstream CUGs (Florkiewicz and Sommer, 1989). The N-terminally extended forms appear to localize predominantly in the cell nucleus. The significance of this finding remains to be elucidated. Also, some forms of acidic FGF are found in the nucleus, but this localization does not appear to require the synthesis of higher molecular weight forms (Imamura *et al.*, 1990).

An interesting mechanism of regulation of bFGF expression has been reported in *Xenopus* oocytes (Kimelman and Kirschner, 1989). In addition to a transcript encoding bFGF, an antisense transcript is present in large excess. The antisense transcript hybridizes to bFGF mRNA, but surprisingly does not appear to inhibit its translation and could be involved in regulation of bFGF mRNA stability. As mentioned above, aFGF and bFGF are not secreted. Thus, a further control on their expression could be exerted at the level of cell release.

INT-2 transcription appears also to be restricted to specific steps of development, but not much is known about its regulation. As mentioned above, INT-2 is expressed in EC cells only after induction of differentiation (Jakobovits *et al.*, 1986; Mansour and Martin, 1988). Regulation appears to be mainly transcriptional, and the combination of three distinct promoters and two alternative polyadenylation sites generates six different RNA species, which, however, all have the same coding capacity (Grinberg *et al.*, 1991; Smith *et al.*, 1988). The main cis-acting elements necessary for INT-2 transcription appear to map to a 1.7-kb fragment of INT-2 DNA, which includes the three promoters, and about 1 kb of upstream sequences (Grinberg *et al.*, 1991). Translation of INT-2 RNA can begin at two sites: from an AUG that produces a secreted protein (although apparently not very efficiently) and from an upstream CUG. The product of upstream initiation partially localizes to the cell nucleus (Acland *et al.*,1990). In addition, INT-2 RNA contains an out-of-frame AUG in its 5′ region, and this AUG apparently interferes with correct translation initiation (Dixon *et al.*, 1989). Thus it appears that INT-2 expression is controlled at at least two main levels: transcription and translation initiation.

The human FGF-5 gene is transcribed into two main RNA species of 1.6 and 4 kb, likely to result from the use of alternative polyadenylation sites (Zahn *et al.*, 1988). The regulatory elements of FGF-5 transcription are not yet identified, but there is evidence of translational control. The

FGF-5 mRNA contains a short out-of-frame open reading frame (ORF) upstream of the ORF coding for the growth factor. Deletion of the upstream ORF enhances FGF-5 translation and transforming ability (Bates *et al.*, 1991). FGF-5 is efficiently secreted as a glycosylated protein.

Not much is known about regulation of FGF-6 and KGF expression. They both appear to be secreted proteins. In conclusion, regulation of FGF expression is complex and takes place at many levels. The data available so far are compatible with the hypothesis that release of these very potent and broad spectrum mitogens must be tightly regulated physiologically. Thus K-FGF has a very tight transcriptional control. Basic and acidic FGF, which are more ubiquitously transcribed, have a complex regulation of translation and are normally not secreted. INT-2 expression is controlled transcriptionally and translationally. Perhaps the broader the spectrum of action of the growth factors, the tighter is the regulation of their production.

IV. FGF Receptors

The discovery of seven growth factors, many of which seem to have a very similar spectrum of action, raises the question of what could be the evolutionary advantage for the organism in producing many growth factors with similar target specificity. While a partial answer to this question can be glimpsed from the pattern of expression of these genes, which is quite different, the final answer will require the identification and characterization of FGF receptors, their tissue distribution, and specificity of interaction with the various members of the FGF family.

A number of laboratories have studied binding of basic and acidic FGFs to cellular plasma membranes. These studies led to the conclusion that these growth factors bound to two types of cell surface receptors. A low-affinity receptor, which is widely distributed with a large number of sites per cell ($1-2 \times 10^6$) and with a binding affinity of 2 to 10 nM. These receptors are likely to be heparan sulfate proteoglycans (HSPGs). In addition, studies demonstrate the presence of high-affinity receptors ($K_d \sim 10-100$ pM), with a lower number of sites per cell (10,000–100,000) and a molecular weight on the order of 110,000–160,000. These receptors are glycosylated proteins with intrinsic tyrosine kinase activity, a characteristic of many growth factor receptors (reviewed in Baird and Bohlen, 1990; Burgess and Maciag, 1989; Rifkin and Moscatelli, 1989).

The elucidation of the nature of these receptors has taken great impulse from the first cloning of the complete cDNA for an FGF receptor, which was performed by Lee *et al.* (1989) after receptor purification from chicken embryos. This receptor turned out to be highly homolo-

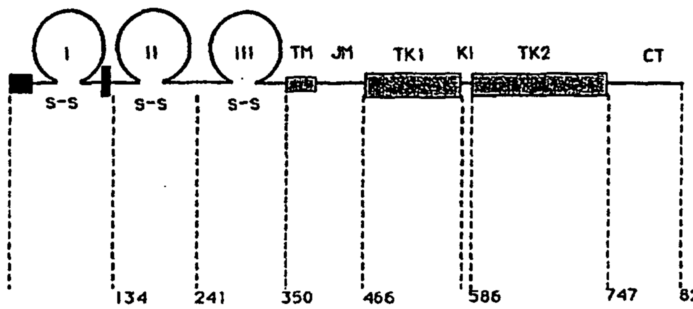
gous to the protein encoded in a partial cDNA (*flg*) isolated from a human endothelial cell cDNA library by its homology to a tyrosine kinase receptor (Ruta *et al.*, 1988). Another previously identified partial cDNA of mouse origin, *bek*, also isolated on the basis of its encoding a tyrosine kinase (Kornbluth *et al.*, 1988), was quickly seen to have substantial homology to the Flg receptor.

Subsequent or concurrent efforts by a number of laboratories have led to an explosion in this field (Dionne *et al.*, 1990; Hattori *et al.*, 1990; Johnson *et al.*, 1990; Keegan *et al.*, 1991; Mansukhani *et al.*, 1990; Musci *et al.*, 1990; Partanen *et al.*, 1991; Pasquale, 1990; Pasquale and Singer, 1989; Ruta *et al.*, 1989; Safran *et al.*, 1990), with results that at present can be summarized in the following manner: the high-affinity FGF receptors also constitute a gene family, which includes at least four members. They share a common structure consisting (from the N terminus to the C terminus) of a signal peptide, three immunoglobulin-like loops flanked by characteristic cysteines, and a hydrophobic transmembrane region. There is a characteristic acidic region between the first and second immunoglobulin (Ig) loop. The intracellular domain includes the catalytic tyrosine kinase domain, which is split by a short kinase insert, as is present in the platelet-derived growth factor (PDGF) receptor (reviewed in Ullrich and Schlessinger, 1990), and a rather long C-terminal tail (Fig. 3).

The degree of homology of the receptors varies, with the highest homology in the tyrosine kinase domain, and the lowest in the first Ig loop (Fig. 3). The nomenclature of these receptors is starting to be as confusing as that of the FGFs, reflecting the isolation of these clones (sometimes fortuitous), the species of origin, etc. Thus, FGF-R1 has been called Flg, Cek-1, etc. FGF-R2 has been called Bek, Cek-3, etc. FGF-R3 has been called also Cek-2. FGF-R4 possibly has no other name.

The primary transcripts of these genes are unusually prone to alternative splicing (Hou *et al.*, 1991). Thus cDNAs encoding soluble receptors, truncated receptors, Ig loop variants, etc., have been isolated (Johnson *et al.*, 1990). Their physiological and pathological significance remains to be elucidated and with some exceptions will not be discussed here. Since these receptors are often coexpressed in tissue culture cell lines, it becomes clearly necessary to express each molecule in receptor-negative cells to test their specificity of binding, receptor activation, etc. To date, the following facts have emerged.

1. The FGFR-1 (Flg) has been expressed in Chinese hamster ovary (CHO) cells, NIH 3T3 cells, and in *Xenopus* oocytes (Dionne *et al.*, 1990; Johnson *et al.*, 1990; Mansukhani *et al.*, 1990). Chinese hamster ovary



% identity									overall identity
FGFR-1/FGFR-2	38	81	79	66	88	50	92	60	71
FGFR-1/FGFR-4	22	63	74	32	75	14	86	38	56
FGFR-1/FGFR-3	21	66	82	41	83	43	91	44	61
FGFR-2/FGFR-4	21	70	72	31	78	29	84	48	57
FGFR-2/FGFR-3	27	72	81	45	87	50	92	56	65
FGFR-3/FGFR-4	26	78	74	34	78	36	86	46	60

FIG. 3. Structure of the FGF receptors and protein homology among the various domains of the four human FGF receptors known. The "wild-type" form of receptors is shown. Variant forms produced by alternative splicing are not shown. Amino acid numbers on the schematic are those for human FGFR-1 (flg). Loops I–III represent the extracellular Ig-like domains. TM, transmembrane region; JM, juxtamembrane region; TK, tyrosine kinase domains; KI, kinase insert; CT, carboxy-terminal tail. Stippled box, signal peptide; dark box, acidic region.

cells are essentially FGF receptor negative, although some differences between the various CHO strains and clones are likely to exist. NIH 3T3 cells are receptor positive and necessitate large levels of expression. Testing in *Xenopus* oocytes (following microinjection of RNA) allows measuring calcium effluxes in response to FGFs, but does not allow measurements of binding affinity, etc. FGFR-1 appears to bind acidic and basic FGF with similar high affinity and K-FGF with about 15-fold lower affinity (Dionne *et al.*, 1990; Mansukhani *et al.*, 1990). The receptor kinase is activated following growth factor binding and a significant proliferative response is obtained. Results in other systems confirm these conclusions. Interestingly, a murine Flg receptor lacking the first immunoglobulin loop behaves in this assay like the wild-type receptor (Mansukhani *et al.*, 1990), indicating that this domain of the protein is not important for FGF binding or receptor activation.

2. The FGFR-2 (Bek) cDNA has also been expressed in CHO and NIH 3T3 cells (Dionne *et al.*, 1990; Mansukhani *et al.*, 1992). It appears to bind aFGF, bFGF, and K-FGF with similar high affinity. Interestingly, the recently described KGF receptor (Miki *et al.*, 1991) appears to be a

Bek variant that differs from prototype Bek in lacking the first Ig-like domain and the acidic region and considerably diverges in the third Ig loop. This receptor appears to bind KGF and acidic FGF with high affinity, but binds bFGF with about 20 times lower affinity (Miki *et al.*, 1991). Considering what was said above, this strongly points to the third Ig loop as an important domain for growth factor binding.

3. The third and fourth cDNAs encoding distinct but related FGF receptor molecules have been isolated by screening cDNA libraries from K562 erythroleukemia cells for tyrosine kinases (Keegan *et al.*, 1991; Partanen *et al.*, 1991). This was somewhat surprising, since hematopoietic cells are generally not thought to respond to FGFs, but is in agreement with the finding that human hematopoietic stem cells respond to basic FGF with increased survival and proliferative ability (Gabbianelli *et al.*, 1990). FGFR-3 is found expressed in brain, lung, and kidneys, while FGFR-4 is prevalent in adrenals, lung, and pancreas. A detailed analysis of binding specificities of FGFR-3 and -4 is not yet available. Preliminary results indicate that both FGFR-3 and -4 bind acidic FGF with higher affinity than basic FGF (Partanen *et al.*, 1991; D. Ornitz, personal communication).

Although these results are still rather preliminary, they clearly suggest a complex picture of many receptors with overlapping specificity of binding, yet with preferential affinity for one ligand or another. The physiological significance of a low binding affinity (e.g., FGFR-1 for K-FGF) remains unknown in the absence of a precise knowledge of what are the real concentrations of these growth factors in tissues and organs. It could represent a purely evolutionary conservation of structure, with no physiological implications: in other words, in the example chosen, Flg would not be the physiological target for K-FGF. On the other hand, this could be another mechanism of regulation of growth factor action, with activation taking place only at relatively high growth factor concentrations.

The interpretation of the final effectiveness of one or another of the FGFs vis à vis their affinity for a specific receptor is complicated by what has recently emerged on the low-affinity receptors. Until recently the function of these receptors was substantially unknown. A variety of biochemical evidence indicated that they consisted of heparan sulfate-like glycosaminoglycans, present on the cell surface and in the extracellular matrix. It was also known that binding to low-affinity receptors could be competed by high concentrations of soluble heparin. Since heparin stabilizes and somewhat potentiates the action of most FGFs, an essential role of low-affinity receptors in receptor activation and signal transduc-

tion was generally not postulated. The situation has changed dramatically during the last year, as several lines of evidence emerged to indicate an essential role of low-affinity receptors in FGF action. Barr's group reported the cloning of a low-affinity hamster receptor (Kiefer *et al.*, 1990). The predicted protein structure is that of an HSPG with a short cytoplasmic tail. Expression of this molecule can make lymphoid cells capable of binding to FGF-coated dishes. Interestingly, the protein is highly homologous to the mouse syndecan HSPG, which has been implicated in cell adhesion and other processes (Saunders *et al.*, 1989). The primary amino acid sequence predicts a proteolytic cleavage site separating most of the extracellular portion from the transmembrane and cytoplasmic regions. Thus this portion of the molecule could become part of the ECM.

Evidence pointing to the importance of this or similar molecules comes from a diverse set of experiments. When the Flg receptor was transfected into CHO cell mutants deficient in the production of heparan sulfates, it was found that these cells did not bind bFGF (while wild-type cells did), but could bind bFGF in the presence of heparin (Yayon *et al.*, 1991). It was also shown that treatment of various cell types with heparitinase or culture in the presence of sodium chlorate, which blocks sulfation, resulted in drastic reduction of FGF binding to its receptors, and again binding was restored by the presence of heparin (Rapraeger *et al.*, 1991). In addition, transfection of the Flg or Bek receptor into 32D cells, an IL-3-dependent murine hematopoietic cell line that does not express significant levels of HSPG, creates cells that now can grow in the presence of bFGF and K-FGF, but only in the presence of heparin. Low doses of heparin are sufficient (0.5–1 μg) to show this effect and it can be shown that the action of heparin does not consist in stabilization of FGF against proteolytic degradation (Mansukhani *et al.*, 1992). All these results suggest that FGFs must interact with low-affinity receptors in order to be able to activate the high-affinity receptors, and that the role of low-affinity receptors can be replaced by heparin. The fact that soluble heparin can substitute for the low-affinity receptors suggests that it modifies somewhat the structure of the growth factor and makes it functionally capable of binding and activating the high-affinity receptors (Fig. 4). It does not support the hypothesis that low- and high-affinity receptors oligomerize and create a higher affinity structure.

Much about this novel mechanism of "presentation" of a growth factor to its receptors remains to be elucidated. The nature and diversity of low-affinity receptors will surely be investigated. It will have to be conclusively demonstrated that the heparin requirement in the systems de-

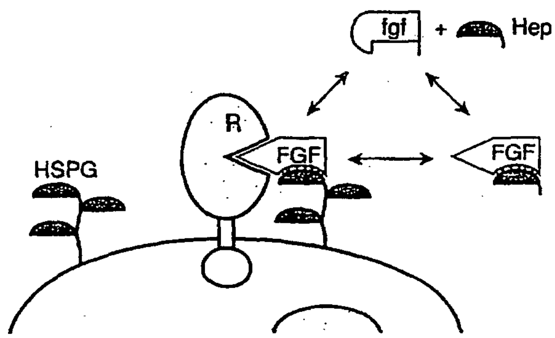


FIG. 4. bFGF interaction with heparin (Hep) is necessary for its binding to receptors. The diagram represents the proposal that bFGF in the absence of heparin exists in a conformation that is not able to interact with its receptor. Interaction with heparin in solution or heparan sulfates (HSPG) on the cell surface convert the bFGF to a conformation that is recognized by the receptor. [Reprinted from Yayon *et al.* (1991).]

scribed above can be alleviated by the expression of low-affinity receptors. Finally, it is evident from what is discussed above that modifications of FGF "potency" could be achieved by a factorial combination of their ability to bind low- and high-affinity receptors. The modification of FGF structure or function following heparin binding, and how that affects receptor binding, will have to be studied. Much of this work will probably require studying the crystal structure of FGFs with or without heparin, that of the extracellular domain of the receptors, etc.

The mechanism of signal transduction by FGF receptors is only beginning to be studied. Like many other receptor tyrosine kinases, FGF receptors probably oligomerize following ligand binding (Ullrich and Schlessinger, 1990; Yarden and Ullrich, 1988), resulting in activation of tyrosine kinase activity and trans- and autophosphorylation. One of the substrates of the FGF receptor tyrosine kinase appears to be phospholipase Cγ and a 90-kDa protein can also be easily detected in mouse cells by anti-phosphotyrosine antibodies following receptor activation (Burgess *et al.*, 1990b; Coughlin *et al.*, 1988). The nature of the 90-kDa protein is still unknown. The significance of the frequent coexpression of some of the receptors (e.g., Flg and Bek) is yet unknown. Recent evidence indicates that different FGF receptors can create heterodimers following ligand binding (Bellot *et al.*, 1991). It will be interesting to know whether this leads to different patterns or efficiency of activation, i.e., whether coexpression of two distinct receptors with similar affinity for a given growth factor leads to different patterns of oligomerization and receptor activation and whether that in any way modifies or regulates the interaction of activated receptors with their substrates.

V. Interaction with Extracellular Matrix

Although signal transduction occurs through binding to receptors, the actions of FGFs are also influenced through their interactions with extracellular matrix. As expected from its high affinity for heparin, bFGF added exogenously to cultured cells binds to heparin-like molecules produced by the cells (Baird and Ling, 1987; Moscatelli, 1987; Vlodavsky *et al.*, 1987). These molecules have been identified as HSPGs present on the cell surface and in the extracellular matrix (Saksela *et al.*, 1988; Saksela and Rifkin, 1990). bFGF interacts with the heparan sulfate moieties of these molecules (Saksela *et al.*, 1988) and efficient binding depends on the presence of *N*-sulfate groups (Bashkin *et al.*, 1989). In endothelial cell cultures, the cell surface and extracellular matrix bFGF-binding HSPGs appear to be distinct molecules, the cell surface form having a molecular weight of 250K and the matrix-associated form having a molecular weight greater than 800K (Saksela and Rifkin, 1990). As mentioned above, the cell surface bFGF-binding HSPG of hamster kidney cells was recently cloned and was found to be homologous to the mouse HSPG syndecan (Kiefer *et al.*, 1990). Cloned syndecan has also been shown to bind bFGF. However, syndecan is probably not the only cell surface HSPG that binds bFGF, and other bFGF-binding HSPGs are likely to be present in other cell types. bFGF has also been shown to bind to heparin-like molecules in basement membranes *in vivo* (Jeanny *et al.*, 1987) and to be present in isolated basement membranes (Folkman *et al.*, 1988), providing further evidence that bFGF-binding HSPGs exist in addition to the cell surface ones.

bFGF has a lower affinity for these heparan sulfates ($2\text{–}600 \times 10^{-9} M$) than for its cell surface high-affinity receptors ($2\text{–}5 \times 10^{-11} M$) (Bashkin *et al.*, 1989; Dionne *et al.*, 1990; Moenner *et al.*, 1986; Moscatelli, 1987; Vigny *et al.*, 1988). Binding to the HSPGs does not preclude bFGF from binding to receptors (Moscatelli, 1987, 1988). Rather, interaction with HSPGs seems to confer specific biological advantages to bFGF. First, the HSPG-bound bFGF can act as a reservoir of growth factor for the cells. For endothelial cells, it has been shown that when the cells are exposed briefly to 10 ng/ml bFGF, most of the growth factor binds to HSPGs. If the growth medium is changed, leaving the cells with only the HSPG-bound bFGF, biological responses measured 24 hr later are the same as in cultures continuously exposed to bFGF-containing medium. However, if bFGF is stripped from its HSPG-binding sites, biological responses measured 24 hr later are greatly reduced (Flaumenhaft *et al.*, 1989). These results suggest that cells can use the HSPGs as a temporary storage site for bFGF and can draw on this reserve of growth factor to

greatly increase the response to a brief exposure to bFGF. Second, bFGF bound to heparin or heparan sulfates is protected from thermal denaturation and proteolytic degradation (Gospodarowicz and Cheng, 1986; Saksela *et al.*, 1988; Sommer and Rifkin, 1989). Thus, HSPG-bound bFGF is more stable. Third, soluble heparan sulfates can act as carriers for bFGF, increasing its radius of diffusion. Since bFGF interacts strongly with HSPGs fixed in place on the surface of cells and in the extracellular matrix, it does not diffuse freely from its site of release (Flaumenhaft *et al.*, 1990). Soluble heparan sulfates bind bFGF, neutralizing its interaction with fixed HSPGs and increasing its availability to cells distant from its site of release (Flaumenhaft *et al.*, 1990). Finally, as described above, Yayon *et al.* (1991) showed in an elegant series of experiments that bFGF interaction with heparin or heparan sulfates is necessary for its interaction with receptors. The simplest interpretation of these experiments is that binding to heparin or heparan sulfates alters the conformation of bFGF so that it can then interact with its binding site on the receptor.

Since all members of the FGF family bind strongly to heparin, it is likely that other members of the family also interact with heparin-like molecules in the extracellular matrix. Indeed, aFGF has also been shown to bind to extracellular matrix molecules produced by cells (Gordon *et al.*, 1989; Kan *et al.*, 1988) and to basement membranes *in vivo* (Jeanny *et al.*, 1987). Furthermore, more than other members of the family, the biological activity of aFGF is greatly protentiated by the addition of heparin (Mueller *et al.*, 1989; Schreiber *et al.*, 1985; Uhlrich *et al.*, 1986) or HSPGs (Gordon *et al.*, 1989). It is not entirely clear how this potentiation occurs, but it could be related to some of the effects observed for the interaction of bFGF with heparin and heparan sulfates. Like bFGF, aFGF is protected from thermal denaturation and proteolytic degradation by heparin (Gospodarowicz and Cheng, 1986; Rosengart *et al.*, 1988) and therefore may be more stable in a complex with heparin or HSPG. In addition, interaction of aFGF with heparin seems to alter the conformation of the protein since addition of heparin increases the binding of aFGF to specific monoclonal antibodies (Schreiber *et al.*, 1985). This change in conformation may also be responsible for the approximately twofold greater affinity of aFGF for its receptor in the presence of heparin (Kaplow *et al.*, 1990; Schreiber *et al.*, 1985).

The interaction of other members of the family with extracellular matrix molecules has not been studied in detail. Interactions with the extracellular matrix may be especially important in regulating the action of the secreted members of the FGF family. Despite their efficient release from cells, interactions with the extracellular matrix may restrict the

distribution of these factors to areas in the immediate vicinity of the producing cell and thereby modulate their activity.

VI. Biological Function

bFGF and aFGF are potent mitogens for a variety of cells of mesodermal, ectodermal, and endodermal origin (Gospodarowicz *et al.*, 1987). However, the absence of a signal sequence for secretion in these molecules makes it difficult to understand their role and mode of action *in vivo*. How are these molecules released to exert their effects *in vivo*? It has been proposed that they are released from dead or dying cells and, thus, may be primarily involved in responses to tissue destruction. In a variation on this, it has been suggested that bFGF is released through small, nonlethal disruptions of the plasma membrane (McNeil *et al.*, 1989). However, there is increasing evidence that, despite the lack of a signal sequence, low levels of the growth factor are released by healthy cells. In cultured endothelial cells, which both synthesize and have receptors for bFGF, basal levels of protease production and DNA synthesis are inhibited by neutralizing antibodies to bFGF (Sakaguchi *et al.*, 1988; Sato and Rifkin, 1988). These results suggest that the cells release small amounts of bFGF that can activate their own FGF receptors in an autocrine manner. An alternative explanation is that, in mass culture experiments, the death of a minute percentage of the cells releases enough bFGF to cause these results and that healthy cells release no bFGF. This possibility was addressed in experiments in which the migration of single cells expressing different amounts of bFGF was investigated (Mignatti *et al.*, 1991). Cell movement was shown to be proportional to the content of bFGF and could be inhibited by antibodies to bFGF, indicating that the cells were responding to their own bFGF, which was released to a space accessible to the antibodies. Since only a single cell was present in each well during this experiment, the bFGF had to be released from the responding cell. Thus, it appears that healthy cells are able to release small but biologically significant amounts of bFGF. The mechanism of bFGF release is still unclear.

One of the major roles proposed for the FGFs *in vivo* is in the induction of new blood vessel growth or angiogenesis (Folkman and Klagsbrun, 1987). Angiogenesis occurs physiologically in the development of the vascular system during embryonic, fetal, and adolescent growth, and in the growth of the uterine lining during the menstrual cycle. Angiogenesis also contributes to several pathologies either directly, as in diabetic retinopathy, or indirectly by supporting the growth of pathologic tissues, as in rheumatoid arthritis and tumor growth. Neovasculariza-

tion occurs from capillaries and is initiated when the capillary endothelial cells break through their basement membranes, migrate toward the source of angiogenic inducer, and proliferate, forming new cords of endothelial cells that eventually develop into capillaries (Ausprunk and Folkman, 1977). The FGFs have effects on cultured endothelial cells that are consistent with a role in this process. bFGF has been shown to induce an invasive phenotype in cultured endothelial cells, enabling them to penetrate basement membranes *in vitro* (Mignatti *et al.*, 1989). The ability to penetrate the basement membrane is dependent on the increased production of the proteolytic enzymes plasminogen activator and collagenase in response to bFGF (Mignatti *et al.*, 1989; Moscatelli *et al.*, 1986b; Presta *et al.*, 1986). aFGF and K-FGF are likely to have similar effects, since they also stimulate the production of proteolytic enzymes in cultured endothelial cells (Delli Bovi *et al.*, 1988). In addition, both aFGF and bFGF are chemotactic for endothelial cells (Moscatelli *et al.*, 1986b; Terranova *et al.*, 1985), suggesting that these factors support the directed growth of capillaries during angiogenesis. Finally, aFGF, bFGF, K-FGF, and FGF-5 stimulate endothelial cell proliferation (Delli Bovi *et al.*, 1988; Gospodarowicz *et al.*, 1987; Zhan *et al.*, 1988). Thus, members of the FGF family have properties expected of angiogenic factors, and, indeed, aFGF and bFGF have been shown to induce angiogenesis *in vivo* in a number of model systems (Hayek *et al.*, 1987; Lobb *et al.*, 1985; Shing *et al.*, 1985; Thomas *et al.*, 1985).

However, the roles of the FGFs *in vivo* have been difficult to sort out, not only because of the overlapping biological properties of the members of the FGF family, but also because similar biological effects are also induced by unrelated growth factors. Because of their initial isolation as fibroblast growth factors and angiogenesis factors, bFGF and aFGF have been proposed to have a major role in wound healing, especially in the formation of granulation tissue and the accompanying neovascularization. This hypothesis was supported by experiments that showed that application of exogenous bFGF increased granulation tissue formation and the breaking strength of incisional wounds (Davidson *et al.*, 1985; McGee *et al.*, 1988). Furthermore, bFGF greatly improved the normally impaired healing of dermal wounds in diabetic mice, restoring the response to levels seen in normal littermates (Tsuboi and Rifkin, 1990). However, these experiments, like the experiments demonstrating angiogenic effects of purified FGFs, show only that bFGF and aFGF have the capacity to promote wound healing and angiogenesis. Other growth factors have also been shown to have wound healing and angiogenic properties (Folkman and Klagsbrun, 1987), and it is not clear which factors are involved in the natural processes. Indeed, one study has

suggested that bFGF is not involved in some instances of tumor angiogenesis, since high-level expression of antibodies to bFGF in mice did not reduce the angiogenesis of tumors in these animals (Matsuzaki *et al.*, 1989). The role of endogenous bFGF in wound healing and angiogenesis has been addressed by Broadley *et al.* (1989). In these experiments, collagen sponges were implanted subcutaneously in rats, and the effect of antibodies to bFGF on the subsequent formation of granulation tissue in these sponges was investigated. When a pellet that slowly released neutralizing antibody to bFGF was included in the sponge, vascularization of the sponge and granulation tissue formation were inhibited. Thus, endogenous bFGF seems to be required for wound repair and neovascularization in at least some instances.

The FGFs also seem to have important functions in early development. bFGF has been shown to behave as an embryonic inducer in early *Xenopus* embryos. Induction of mesoderm is a result of signals generated from the endoderm. When the animal pole region is dissected away from a *Xenopus* blastula, the isolated animal pole forms only ectoderm. If the animal pole tissue is incubated with bFGF, mesodermal structures are formed (Kimelman *et al.*, 1988; Slack *et al.*, 1987). bFGF is expressed in the oocyte and early embryo and receptors that recognize bFGF are present in the blastula, consistent with its proposed role in early development (Gillespie *et al.*, 1989; Kimelman *et al.*, 1988; Slack and Isaacs, 1989). However, aFGF,K-FGF, and INT-2 also are able to induce mesodermal structures (Grunz *et al.*, 1988; Paterno *et al.*, 1989; Slack *et al.*, 1988), and one of these molecules, especially one of the secreted family members, may be the actual inducer *in vivo*. *Xenopus* blastulas produce other potent mesoderm inducers, called activins, that are members of the transforming growth factor β (TGF-β) family (Thomsen *et al.*, 1990).

While the FGF family member that acts as the physiological inducer has not yet been identified, an elegant demonstration of the importance of FGFs in the formation of mesoderm in *Xenopus* embryos has just been provided (Amaya *et al.*, 1991). Expression of a truncated, tyrosine kinase-deficient FGF receptor in *Xenopus* embryos makes them unable to induce mesoderm in response to FGFs and causes specific defects in gastrulation and development. The truncated FGF receptor can be shown to act as a dominant negative mutant that abolishes wild-type receptor function. Thus FGFs not only can induce mesoderm in *Xenopus*, but their signaling pathway appears to be essential in the formation of posterior and lateral mesoderms.

Perhaps another indication that FGFs are involved in cell differentiation is the report that aFGF causes bladder carcinoma cells to lose their epithelial character and to acquire some properties typical of mes-

enchymal cells (Valles *et al.*, 1990). On exposure to aFGF, the bladder carcinoma cells lost their desmosomal contacts and transformed into elongated fibroblastoid cells. This shape change was accompanied by a decrease in cell surface staining for desmosomal proteins and an induced expression of vimentin intermediate filaments (Boyer *et al.*, 1989; Valles *et al.*, 1990). Interestingly, bFGF was ineffective in altering the morphology of the bladder carcinoma cells, suggesting that these cells express a receptor that recognizes aFGF but not bFGF.

The idea that the FGFs may have important functions in development is also supported by the highly tissue- and time-specific expression of members of this family during murine embryonic growth. At least two of the secreted members of the family, INT-2 and K-FGF, seem to be present only in the embryo or fetus and are not found in normal adult tissue. INT-2, K-FGF, and FGF-5 are expressed at precise steps of development: high levels of K-FGF are detected only in very early embryos, and INT-2 and FGF-5 are produced at later stages of embryonic and fetal growth, each in specific tissues (Hebert *et al.*, 1990; Wilkinson *et al.*, 1988, 1989). The secreted members of the FGF family may act as differentiation signals at specific steps in development.

bFGF is expressed at high levels at later stages of embryonic and fetal development (Hebert *et al.*, 1990) and has been implicated in some differentiation processes. For example, several lines of evidence suggest that bFGF may be involved in muscle differentiation. In the chicken embryo, bFGF is abundant in the myocardium, somite myotome, and developing limb bud muscle (Joseph-Silverstein *et al.*, 1989). In the mouse fetus, bFGF is also detected in high levels in cardiac, skeletal, and smooth muscle (Gonzalez *et al.*, 1990). The amount of bFGF present decreases as the tissues mature (Joseph-Silverstein *et al.*, 1989). This seems to correlate with the observation that cultured myocytes remain undifferentiated when they are maintained in bFGF-containing medium, but differentiate into myotubes on withdrawal of bFGF (Clegg *et al.*, 1987). Differentiation into myotubes is accompanied by a decrease in bFGF and aFGF mRNA expression (Moore *et al.*, 1991). In addition, on differentiation, the expression of FGF receptors is down regulated (Moore *et al.*, 1991; Olwin and Hauschka, 1988). Nevertheless, it is still unclear whether the high levels of bFGF in developing muscle are directly involved in stimulating the proliferation of myocytes or are simply a reflection of higher expression of bFGF mRNA in myocytes than in myotubes.

The FGFs may also be involved in the differentiation and maintenance of nervous tissue. Both aFGF and bFGF as well as K-FGF cause rat PC12 pheochromocytoma cells to send out neurites and to differentiate

into sympathetic neuron-like cells (Rydel and Greene, 1987; Schubert *et al.*, 1987; Togari *et al.*, 1983, 1985; Wagner and D'Amore, 1986). In quail embryos, bFGF is expressed by young neurons of the neural tube and the neural crest, and, at later stages, by neurons of the spinal cord and dorsal root ganglia (Kalcheim and Neufeld, 1990). In the spinal cord, bFGF levels reach a peak on embryonic day 10 and then decrease by hatching time (Kalcheim and Neufeld, 1990), suggesting that bFGF may be important in neural cell movements and formation of connections in the embryo. However, expression of aFGF, bFGF, and FGF-5 persists in adult brain, and brain contains much higher levels of mRNA for these growth factors than other adult tissues (Alterio *et al.*, 1988; Haub *et al.*, 1990; Shimasaki *et al.*, 1988), suggesting that the FGFs are also important in neural physiology in adults. High-level expression of aFGF and bFGF and low-level expression of FGF-5 is limited to neurons in localized ares of the brain. Interestingly, aFGF and bFGF are expressed differently in neighboring areas of specific brain regions: aFGF is expressed in fields CA1 and CA3 of the hippocampus, while bFGF is expressed in field CA2; aFGF is preferentially localized in layers 3 and 5 of the cerebral cortex, while bFGF is expressed in layers 2 and 6 (Emoto *et al.*, 1989; Wilcox and Unnerstall, 1991). In addition to being localized to specific neurons, both aFGF and bFGF seem to be expressed by glial cells (Emoto *et al.*, 1989; Wilcox and Unnerstall, 1991). Both neural and glial-derived FGFs may have important neurotrophic effects on surrounding neurons. These growth factors have been shown to support the survival in culture of neurons isolated from numerous sites in the central nervous system, including the hippocampus, cerebral cortex, ciliary ganglion, spinal cord, and cerebellum (Hatten *et al.*, 1988; Morrison *et al.*, 1986; Unsicker *et al.*, 1987; Walicke *et al.*, 1986). Furthermore, the ability of glial cells to support the survival of neurons in culture has been shown to be due to glial-derived bFGF (Hatten *et al.*, 1988). Finally, the FGFs have been shown to promote the survival of lesioned nerves *in vivo* (Anderson *et al.*, 1988). These results have been interpreted as evidence that the endogenous FGFs in the brain may be necessary for the survival of intact neurons *in vivo* or in establishing neuronal contacts.

VII. Oncogenic Potential

It has become almost obvious that any gene encoding a growth factor has the potential to be an oncogene. Constitutive expression of such a gene in a cell that expresses specific receptor(s) for the growth factor can create an autocrine growth loop, resulting in self-sustained aberrant growth. It is therefore not surprising that three members of the FGF

family were originally identified as oncogenes. INT-2 was identified as a gene frequently activated in mouse mammary carcinomas. K-FGF/HST and FGF-5 were originally isolated as genes capable of transforming NIH 3T3 cells in culture. Cloning of their cDNAs revealed the homologies of their predicted gene products to FGFs. The transforming potential of other FGFs is not equally clear, and in most cases the importance of FGFs as "natural" oncogenes, i.e., their involvement in the etiology or progression of "spontaneous" human or animal malignancies, remains to be established. No FGF has yet been found as a retroviral oncogene, i.e., an oncogene acquired by the many naturally occurring transforming retroviruses. Information about the oncogenic potential of the FGFs is variable from member to member. We will discuss first the molecular aspects of their oncogenic potential *in vitro*, and then their involvement in tumors.

A. K-FGF/HST

The human HST/K-FGF gene was isolated first in Japan by transfection of stomach cancer DNA into NIH 3T3 cells, and at about the same time in New York by transfection of DNA from Kaposi's sarcoma (Sakamoto *et al.*, 1986; Taira *et al.*, 1987; Delli Bovi and Basilico, 1987; Delli Bovi *et al.*, 1987). In both cases, the demonstration that the oncogene was activated in the original tumor is still lacking. While efforts at proving this point were probably hampered by the fact that the original DNA used for transfection came from biopsies and not a cultured cell line, it is probably safe to assume that the isolation of this gene was accidental, i.e., the gene was activated during transfection. Irrespective of this point, it is noteworthy that both the HST and K-FGF oncogenes, as originally described, carried DNA rearrangements (Sakamoto *et al.*, 1986; Delli Bovi and Basilico, 1987). These rearrangements were probably important for "activation" of this oncogene.

Dominant oncogenes are "activated" versions of their normal counterpart (i.e., protooncogenes) and the mechanism of activation has been shown to fall into two broad categories; mutations, ranging from point mutations to deletions and fusions with other coding sequences, and changes in the regulation of expression. The mechanism of activation of K-FGF/HST clearly consists of the latter. Cloning and expression of the human protooncogene revealed that the proteins encoded by the oncogene and protooncogene were identical (Yoshida *et al.*, 1987; Delli Bovi *et al.*, 1988). Thus the mechanism of activation must result from changes in the regulation of gene expression. This conclusion is in line with the finding (see above) that K-FGF expression is extremely re-

stricted in normal tissues and organs, being probably limited to early stages of development (Hebert *et al.*, 1990).

It is therefore quite likely that the rearrangement found outside the coding sequences in the original oncogene isolates played a role in activating gene expression. It is likely (Curatola and Basilico, 1990) that this results from the juxtaposition of general enhancer sequences to the K-FGF gene, resulting in ubiquitous expression, but the possibility of deletion of negative regulatory sequences cannot yet be ruled out. A puzzling observation that has been made in several laboratories (Sakamoto *et al.*, 1988; M. Goldfarb, personal communication; A. M. Curatola and C. Basilico, unpublished) is that the cloned K-FGF protooncogene has low but significant transforming ability when transfected into NIH 3T3 cells. In protooncogene-transformed cells the transfected gene is expressed while the endogenous K-FGF gene remains silent. The transforming ability of the K-FGF protooncogene could be due to integration next to cellular enhancer sequences or to the deletion of negative regulatory sequences on cloning or transfection. Neither hypothesis has yet been fully investigated. Curatola and Basilico (1990) found no evidence of the presence of negative regulatory sequences in the 5′ portion of the K-FGF oncogene when these DNA regions were used to drive CAT expression in a transient assay.

Transformation by K-FGF/HST appears to result from the creation of an autocrine growth loop in which constitutive expression of K-FGF in a cell that also expresses its receptors leads to secretion of the growth factor, activation of the receptor, and continuous stimulation of its signal transduction pathway (Talarico and Basilico, 1991). Cells are thus constantly induced to proliferate. It appears that secretion of the growth factor is required for transformation, i.e., the mitogenic pathway can be activated only at the cell surface. Anti-K-FGF neutralizing antibodies cause reversion of the transformed phenotype, and inhibit the proliferation of K-FGF-transformed cells in serum-free media. To investigate the question of why the phenotype of some K-FGF transformed lines was only partially reverted by antibodies, as well as to test the hypothesis (Keating and Williams, 1988) that interaction of the growth factor with the receptor could also occur intracellularly, Talarico and Basilico (1991) constructed K-FGF cDNA mutants encoding proteins with impaired secretion. These mutants had extremely reduced transforming ability and the rare transformed cells they could produce appeared to secrete minute amounts of the growth factor. Their phenotype was fully reverted by anti-K-FGF antibodies. Thus it appears that intracellular activation of the receptor cannot occur in this system, perhaps because FGFs must interact with heparan sulfates (see above) before activating the receptor.

Alternatively, this interaction could occur, but in such rare circumstances as to have no practical effect on the regulation of cellular proliferation.

B. INT-2

The INT-2 gene was originally identified and cloned as a frequent site of insertion of mouse mammary tumor virus (MMTV) proviral DNA in mouse mammary carcinomas. Insertion of the MMTV long terminal repeat (LTR) upstream or downstream of the INT-2 gene leads to activation of its expression (Dickson *et al.*, 1984; Moore *et al.*, 1986). Here again, therefore, the mechanism of INT-2 oncogenic activation results from induction of transcription, and the gene is not expressed in normal breast tissue (Dickson *et al.*, 1984). Thus induction of INT-2 expression is probably one of the early events (but certainly not the only one; see below) in mouse mammary carcinogenesis.

Our knowledge of the mechanism of transformation by INT-2 is limited, particularly because INT-2 is not a strong transforming gene in tissue culture systems. NIH 3T3 cells can be transformed by INT-2 but transformation is inefficient and appears to require large amounts of the protein (Goldfarb *et al.*, 1991). One of the reasons for this poor efficiency may be the fact that INT-2 is not efficiently secreted, although it possesses a signal peptide (Dixon *et al.*, 1989), possibly because upstream initiation of translation at an in-frame CUG results in the synthesis of a protein with 29 additional amino acids, which seems to localize to the nucleus (Acland *et al.*, 1990). However, mutagenesis of the INT-2 cDNA clearly indicates that only the secreted form of INT-2 is transforming, while no discernible phenotype is associated with the expression of an N-terminally extended protein that has the signal peptide deleted and is almost exclusively localized in the nucleus (Dickson *et al.*, 1991). Thus the general conclusions outlined for K-FGF, i.e., that transformation requires growth factor secretion and extracellular activation of the receptor(s), probably hold true for INT-2 also. It cannot, however, be ruled out that INT-2 is also a weak mitogen for the cells tested so far, possibly because its affinity for their receptor is low. Most of these questions will be answered when large amounts of pure INT-2 protein are available.

C. FGF-5

FGF-5 was also isolated as an oncogene by transfection of DNA from a tumor cell line into NIH 3T3 cells. The mechanism of activation was clearly shown to result from the juxtaposition of enhancer sequences 5′

to the FGF-5 gene. The rearrangement had occurred during transfection as the enhancer sequences were those of the pLTR-Neo plasmid, which had been cotransfected together with tumor cell DNA (Zhan *et al.*, 1988). FGF-5-transfected cells were selected for growth in serum-free medium.

It is thus likely that for FGF-5, as well, activation results from changes in the regulation of expression. This could be due to different mechanisms, as it has been reported that the FGF-5 mRNA contains a short open reading frame, upstream of that coding for the growth factor. Translation of this ORF decreases translation initiation from the AUG of FGF-5 (Bates *et al.*, 1991). Expression of FGF-5 has been detected in several tumor cell lines (Zhan *et al.*, 1988). The significance of this finding remains to be elucidated. FGF-5 is also a secreted protein (Bates *et al.*, 1991), and although not much is known about its mechanism of transformation, it is quite likely that, as in the case of K-FGF and INT-2, transformation requires secretion and activation of the receptor at the cell surface.

D. bFGF AND aFGF

bFGF and aFGF were not identified as oncogenes and to our knowledge they have never been isolated in a transformation assay such as NIH 3T3 transfection. This is not surprising since basic and acidic FGF cDNAs under the control of a constitutive promoter do not display a significant transforming ability in tissue culture (Sasada *et al.*, 1988; Quarto *et al.*, 1989; Rogelj *et al.*, 1988; Jaye *et al.*, 1988; Neufeld *et al.*, 1988). As discussed, these two proteins do not contain a signal peptide and are not efficiently released from producer cells. It is therefore likely that their lack of oncogenic potential in tissue culture stems from lack of secretion. In line with the hypothesis, it has been shown that recombinant bFGF cDNAs in which a sequence encoding a signal peptide had been inserted in the 5′ region can efficiently transform cells in culture (Rogelj *et al.*, 1988; Blam *et al.*, 1988).

A number of papers have reported that high levels of expression of native bFGF or aFGF can confer to NIH 3T3 cells a transformed phenotype, but these cells are generally not tumorigenic in animals (Sasada *et al.*, 1988; Rogelj *et al.*, 1988; Jaye *et al.*, 1988; Quarto *et al.*, 1989). On the other hand, the studies of Wellstein *et al.* (1990) showed that SW-13 adrenal cells, which constitutively produce large amounts of bFGF, do not grow in agar and are not tumorigenic. Growth in agar and tumorigenicity followed transfection with K-FGF cDNA. It can probably be concluded that the oncogenic potential of bFGF and aFGF is very

limited, and can only be manifested when very high levels of expression are achieved.

A related question is whether the transformed phenotype of the rare cells transformed by bFGF and aFGF is due to small amounts of protein that these cells may release. These proteins are not detectable by biochemical methods, but experiments using blocking antibodies have in some cases demonstrated a reversion of the transformed phenotype (Sasada *et al.*, 1988). We consider it therefore likely that transformation by bFGF or aFGF can be achieved, when, due to very high levels of expression of these proteins, a minute amount of growth factor is released into the medium, and can thus activate cell surface receptors. An alternative hypothesis is that this low level of transformation results from the synthesis of FGF molecules that are not released into the extracellular compartments, but are targeted to the nucleus. However, experiments using mutant bFGFs capable of expressing only the N-terminally extended forms associated with nuclear localization have failed to substantiate this hypothesis (Quarto *et al.*, 1991b), and the same was true for INT-2, in which only the secreted forms are capable of transformation (Dickson *et al.*, 1991) (see above).

E. FGF-6 AND KGF

FGF-6 expression can transform NIH 3T3 cells, and apparently also in this case secretion of the growth factor is important (Marics *et al.*, 1989; deLapeyriere *et al.*, 1990). Nothing is known so far about the oncogenic potential of KGF.

VIII. Involvement of FGFs in Tumors

FGFs are generally angiogenic, and thus the first demonstration of the possible involvement of these growth factors in tumor formation or progression was the identification of TAF (tumor angiogenesis factor; later proved to be bFGF) from several tumors (Folkman *et al.*, 1971). Thus the role of FGFs in tumor formation has been investigated mainly from two angles, that of factors necessary to promote tumor vascularization, and as primary oncogenes.

A. INT-2 AND K-FGF

The involvement of INT-2 in mouse mammary carcinomas produced by MMTV infection is quite clear and has been demonstrated in a number of laboratories. As the name indicates, INT-2 was the second

gene clearly identified as a very frequent site of integration of the MMTV proviral DNA in mammary tumors (Dickson *et al.*, 1984; Moore *et al.*, 1986). This does not imply, however, that insertion near INT-2 is a preferential attribute of MMTV, but likely results from random integration events in widespread viral infection, and selection by tumor growth of cells in which the INT-2 gene has been activated. It is not clear whether activation of INT-2 is all that is required for mammary carcinogenesis. Mice transgenic for INT-2 under the control of the MMTV enhancer/promoter develop a high incidence of mammary hypertrophia following pregnancy, presumably reflecting the increased hormonal stimulation of the MMTV regulatory elements (Muller *et al.*, 1990). However, these hypertrophic manifestations generally regress, indicating that INT-2 expression alone is not sufficient for tumor formation.

K-FGF has also been found to be activated by MMTV insertion in some mouse tumors (Peters *et al.*, 1989), and a recent publication suggests that activation of both INT-2 and K-FGF is important for the metastatic ability of these tumors (Murakami *et al.*, 1990).

These and other findings have led investigators to study possible rearrangements or expression of INT-2 in human breast carcinomas. A high percentage (~20%) of these human tumors appear to carry amplifications of both the INT-2 and K-FGF genes (Tsutsumi *et al.*, 1988; Tsuda *et al.*, 1989; Theillet *et al.*, 1989; Adnane *et al.*, 1989; Ali *et al.*, 1989) (this is not surprising, since as already discussed these genes map very close one to another), but with some exception no clear-cut evidence of their expression (at the RNA level) was found. The interpretation of this finding remains to be provided. Since in general gene amplification is not detectable unless selected for, and selection could not be expected to occur without gene expression, it could be that INT-2 and K-FGF are passive bystanders in a large amplification event that involves a yet undiscovered oncogene mapping in their vicinity on chromosome 11. Alternatively, INT-2 and K-FGF amplification could represent a past event in the evolution of the tumors, which is no longer essential for tumor growth because other genetic alterations have taken place. This possibility seems, however, quite unlikely. It should be mentioned that other cases of amplification of INT-2 together with K-FGF have been reported, particularly in squamous carcinomas of the head and neck (Tsutsumi *et al.*, 1988; Theillet *et al.*, 1989; Tsuda *et al.*, 1988). Again, no clear evidence of INT-2 or K-FGF expression has been reported.

In human tumors, K-FGF has been found to be expressed in a number of teratocarcinomas and germ cell tumors (T. Yoshida *et al.*, 1988; Schofield *et al.*, 1991), but the significance of this finding in regard

to the etiology of these tumors is doubtful, as K-FGF is physiologically expressed in undifferentiated embryonal carcinoma cells and in the blastocyst. Thus K-FGF expression in teratocarcinomas is likely to reflect simply their stage of differentiation, rather than being responsible for their tumorigenic phenotype. Amplification has also been found in several tumors, and is discussed above. K-FGF was isolated by transfection of Kaposi's sarcoma (KS) DNA as well as from stomach cancer DNA. As already discussed, however, it is likely that the isolation was coincidental and did not reflect the activation of this oncogene in the original tumors. No evidence has been found so far of K-FGF expression in KS biopsies (A. Friedman-Kien and C. Basilico, unpublished), and although these experiments could have been hampered by the peculiar characteristics of KS, it is unlikely that K-FGF plays an important role in KS. Similar conclusions were reached about stomach carcinomas (Tsuda *et al.*, 1988). Future experiments will undoubtedly answer these questions more conclusively.

On the other hand, K-FGF can clearly be an oncogene in mice. Evidence for this conclusion comes from experiments showing that K-FGF is one of the oncogenes that can be activated by MMTV in mouse mammary carcinomas (Peters *et al.*, 1989), and by the occurrence of a fibrosarcoma in a transgenic mouse that, although carrying a human K-FGF transgene, did not generally express it in any tissue. The tumor, which arose after a long latency, clearly expressed K-FGF RNA and protein (D. Talarico and C. Basilico, unpublished).

Talarico *et al.* (1992) have tried to gain information on the cellular targets of the K-FGF oncogene *in vivo* by constructing a recombinant retrovirus expressing the K-FGF protein. This virus, when injected into newborn immunocompetent mice, originally produced no detectable pathologies, with the exception of long-latency T cell leukemias, due to the helper leukemia virus that had been coinjected to allow multiplication of the defective transforming virus. One animal, however, developed a fibrosarcoma that, when grown in tissue culture, was found to produce high titers of transforming K-FGF virus, together with helper Moloney leukemia virus. This virus was highly tumorigenic when injected into newborn immunocompetent mice, as well as in nude mice. The virus differed from the original construct, and appeared to be the result of a recombination event between the K-FGF-containing retrovirus and the helper. The new virus contained the *gag, pol,* and *env* genes of Mo-MuLV, with the *env* gene fused to the K-FGF cDNA sequences. The resulting protein is an Env–K-FGF fusion protein that maintains most of the K-FGF sequence and ~300 amino acids of Env. It is secreted (presumably utilizing the Env signal peptide) and has a molecular weight

more than double that of native K-FGF. In spite of this drastic rearrangement, this protein appears to have maintained all of the properties of native K-FGF. The pathologies produced by this novel virus in mice are of two types: fibrosarcomas, which generally occur at or near the site of injection, and an unusual form of meningioma, or meningeal fibrosarcoma, originating from the dura mater. These tumors, which have a multifocal (polyclonal) origin, result in massive hyperproliferation of the meninges that surround the brain and the spinal cord. They cause early hydrocephalus, which eventually kills the animal. These intriguing findings raise the possibility that meningeal cells may be exquisitely susceptible to the action of K-FGF, possibly because they express highly specific K-FGF receptors. While further investigations should clarify these issues, these findings clearly underline K-FGF oncogenic potential *in vivo*. How these observations can be translated into similar pathological situations in humans remains to be determined.

B. bFGF AND aFGF

bFGF has been implicated in several human cancers, both as angiogenic factor and as a growth factor capable of sustaining autonomous cell proliferation. bFGF RNA has been found frequently to be highly expressed in malignant melanomas (Halaban *et al.*, 1988a). This, together with the finding that bFGF (but also other FGFs) is a potent mitogen for melanocytes (Halaban *et al.*, 1987, 1988b), has led to the hypothesis that bFGF may be an oncogene whose activation is important in the etiology of human melanomas. Halaban *et al.* (1988a) measured bFGF RNA levels in melanomas and normal melanocytes and found them undetectable in normal melanocytes and detectable, albeit at low levels, in many melanomas. In addition, synthetic peptides that act as bFGF antagonists reduced the growth of melanoma cells in chemically defined medium. Addition of neutralizing antibodies against bFGF, however, had no substantial effect. On the other hand, introduction of a bFGF cDNA into normal murine melanocytes rendered them capable of growth without bFGF in tissue culture, but the cells were not tumorigenic (Dotto *et al.*, 1989). In line with these findings, Becker *et al.* (1989) found that antisense oligodeoxynucleotides targeted against human bFGF mRNA inhibited the proliferation of melanoma cells and their ability to form colonies in soft-agar medium. All of these results point to an important role of bFGF in melanomas. The mechanism of activation of bFGF expression in melanomas or the question of whether extracellular release of bFGF was important was not addressed in these studies.

An elevated level of bFGF RNA has also been found in cell lines derived from Kaposi's sarcoma (Ensoli *et al.*, 1989a,b; Werner *et al.*, 1989a). The levels of RNA corresponding to other growth factors (e.g., IL-1) are also substantially elevated and, in addition, some KS cell lines apparently secrete an unknown heparin-binding mitogen (Ensoli *et al.*, 1989b; Werner *et al.*, 1989b). These findings seem to indicate that KS cells have activated the expression of several cytokines and growth factors. Activation of bFGF production could be related to the high vascularization of these tumors, or could be more directly involved in their growth. On the other hand, production of bFGF is observed in many normal and tumor cell lines, and thus the high levels of expression of many growth factors may reflect the cell of origin of KS (still not conclusively identified) rather than the oncogenic potential of FGF. Furthermore, it must be pointed out that the nature of the KS cell lines has never been clearly established; in other words, it is not totally certain that they are derived from KS cells.

An intriguing observation has been made by Kandel *et al.* (1991), studying the insurgence of dermal fibrosarcomas in mice that have been made transgenic for bovine papilloma virus. These tumors grow through definite stages of progression, which can be classified as mild fibromatosis, aggressive fibromatosis, and finally fibrosarcomas. These two latter stages are highly vascularized. Cell lines derived from the various tumor stages differ in many properties *in vitro* and *in vivo*, but in particular mild fibromatosis cell lines produced large amounts of bFGF that is cell associated, while aggressive fibromatosis and particularly fibrosarcoma lines released most of the bFGF-like activity in the culture medium. Thus transition from benign to a malignant and highly vascularized state seems to be associated with changes in the secretion potential of bFGF. The molecular mechanism by which this phenomenon occurs has not yet been elucidated, and the question of how a molecule that does not possess a signal peptide and that has been shown in many laboratories to be incapable of secretion can be converted to an efficiently secreted protein remains to be answered. Since the identification of bFGF as the secreted growth factor was mainly immunological, it is also conceivable that the secreted bFGF-like activity may not be bFGF, but perhaps a closely related but distinct new member of the FGF family.

C. FGFs and Tumor Angiogenesis

bFGF and other members of the FGF family have also been implicated in tumor vascularization. There is little doubt that solid tumors need angiogenesis to grow in mass, and a variety of classical experiments

have demonstrated that tumors can release angiogenic factors (Folkman and Klagsbrun, 1987). bFGF was one of the first angiogenic factors isolated from tumors, but many other factors with angiogenic properties also are produced by neoplastic cells (Folkman and Klagsbrun, 1987). It is not yet clear which of the factors is most relevant for tumor vascularization. The hypothesis that increased expression of FGF (at the RNA or protein level) is a general characteristic of tumors has not yet been demonstrated, and similarly nothing is known about the mechanism of this activation, if it exists. If FGFs are involved in tumor angiogenesis, in the case of bFGF and aFGF the problem of their inefficient release from producer cells remains to be solved (see, however, the results of Kandel *et al.*,1991, discussed above). FGFs accumulate in the ECM, and perhaps tumor cells possess the ability of mobilizing these growth factors from their storage sites, rather than directly releasing FGFs produced by the cells themselves. Since endothelial cells also produce bFGF, it has been suggested (Schweigerer *et al.*, 1987a) that tumors may secrete a factor that increases bFGF production in endothelial cells, leading to autocrine activation of these cells. Thus, the assessment of the precise involvement of FGFs in tumor vascularization will require further experiments.

The possibility that FGFs may be essential for solid tumor growth, and in general the recognition that angiogenesis is necessary for the increase in tumor mass, has prompted several investigators to study whether substances that are inhibitors of vascularization, or are specific antagonists of growth factors, could be used in cancer therapy. Suramin, a polyanion that strongly inhibits the interaction of FGFs with their receptors (Yayon and Klagsbrun, 1990; Wellstein *et al.*, 1991), has been used in clinical trials on cancer patients and in some cases appears to have had remarkable effects on tumor regression (LaRocca *et al.*, 1991; Walz *et al.*, 1991). Suramin, however, binds to a variety of proteins, including other growth factors, and thus could block autocrine growth loops as well as induction of angiogenesis (Yayon and Klagsbrun 1990; Wellstein *et al.*, 1991; Kim *et al.*, 1991). The elucidation of the respective role of various growth factors in tumor vascularization will certainly be made easier by experiments using specific growth factor antagonists that are being developed at a fast pace.

In conclusion, establishing the precise role of FGFs in tumor formation will require further data. This is not very surprising, since the oncogenic potential of FGFs has been discovered quite recently, and even in the case of oncogenes that have been studied for a longer period of time (e.g., the *ras* family) it is not yet clear whether their activation is specific for certain tumors or is just another factor in an ill-defined

multistep pathway to malignancy. Although it is clear that at least the secreted members of the FGF family have oncogenic potential, a critical role in *in vivo* carcinogenesis has only been established for INT-2 in mouse mammary tumors. The probability that a protooncogene becomes a relevant oncogene is determined by a variety of factors, including the likelihood of its activation in cells that respond to this oncogene, and the probability that other important steps in carcinogenesis, such as the inactivation of a tumor suppressor gene, occur in the same cells. Finally, the host immune response to the phenotype of the cell carrying the activated oncogene may well determine whether such cells will produce a tumor or not. It is quite likely in our view that FGF activation will turn out to be one of the many factors contributing to the malignancy of many solid tumors, but whether specific tumors will turn out to involve FGF activation more than others remains to be determined.

IX. Concluding Remarks

To review a field of research that has undergone a recent "explosion" is fraught with difficulties. Since it would have been impossible to cover all that has been published in the field, we have been trying to concentrate on the aspects of those growth factors that we felt were potentially of higher interest for future development. Without any doubt this will reveal our biases, and we would not be surprised if a number of observations that we overlooked would reveal themselves to have been of great importance in the future years.

The FGF field offers at present a fascinating series of questions to scientists interested in many diverse areas, far and beyond the interest in growth factor action on cell proliferation. Students of regulation of gene expression can find all possible mechanisms operating on the expression of FGFs and their receptors: transcriptional controls, posttranscriptional regulation involving alternative splicing, alternative translation starts resulting in proteins with different properties, and control affecting the secretion of these proteins. Students of development will undoubtedly be attracted by the strong evidence that these growth factors play a role in development, although the precise role is not yet totally clear. Students of angiogenesis and oncogenesis will no doubt be interested in the possible role of FGFs in physiological and pathological angiogenesis, as well as in their oncogenic potential. Finally, the existence of a family of growth factors as well as a family of receptors whose specificity of interaction is only beginning to be elucidated should be of interest to students of signal transduction and of the mechanisms by which growth factors influence cell proliferation, survival, and differentiation. It will

be quite apparent from reading this article that a great many questions in this field remain unanswered. We do not know the exact physiological function of FGFs, we do not know whether their involvement in oncogenesis is only potential or real, we do not know why there are seven growth factors (so far) with an apparently similar spectrum of action. The available data, however, clearly support a number of verifiable hypotheses and thus we are confident that most of these questions will be answered in the years to come.

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